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Structure of the complex of *Neisseria gonorrhoeae* *N*-acetyl-L-glutamate synthase with a bound bisubstrate analog

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ABSTRACT

N-Acetyl-L-glutamate synthase catalyzes the conversion of AcCoA and glutamate to CoA and *N*-acetyl-L-glutamate (NAG), the first step of the arginine biosynthetic pathway in lower organisms. In mammals, NAG is an obligate cofactor of carbamoyl phosphate synthetase I in the urea cycle. We have previously reported the structures of NAGS from *Neisseria gonorrhoeae* (ngNAGS) with various substrates bound. Here we reported the preparation of the bisubstrate analog, CoA-*S*-acetyl-L-glutamate, the crystal structure of ngNAGS with CoA-NAG bound, and kinetic studies of several active site mutants. The results are consistent with a one-step nucleophilic addition–elimination mechanism with Glu353 as the catalytic base and Ser392 as the catalytic acid. The structure of the ngNAGS-bisubstrate complex together with the previous ngNAGS structures delineates the catalytic reaction path for ngNAGS.

1. Introduction

N-Acetyl-L-glutamate synthase (NAGS) catalyzes the first committed step in the arginine biosynthetic pathway in most microorganisms and plants, the acetylation of L-glutamate by AcCoA to produce *N*-acetyl-L-glutamate (NAG) [1,2]. Protein sequence comparison identified two major types of NAGS [3]. The first type, termed classical NAGS or bacterial-like NAGS, identified in most bacteria and plants, has an inactive amino acid kinase (AAK) domain and an active GCN5-like *N*-acetyltransferase (NAT) domain. The second type, termed vertebrate-like NAGS, has been identified in vertebrates, including mammals, and also in fungi and some bacteria. Since mutations in the human NAGS gene cause severe hyperammonia [4] understanding vertebrate-like NAGS has considerable clinical relevance.

Although vertebrate-like NAGS also consists of two domains, AAK and NAT domains, the NAT domain of this enzyme type seems to have evolved from different ancestors than bacterial-like NAGS, since the sequence identity of this domain is as low as 9–20%. Interestingly, fungal *N*-acetyl-glutamate kinases (NAGK), which also belong to this type, have an active AAK domain and an inactive NAT domain [5]. In some bacteria such as *Xanthomonas campestris* and *Maricaulis maris*, the two domains have both NAGS and NAGK catalytic activity, respectively. These enzymes are referred to as bifunctional NAGS/K [3,6], and they are thought to be the ancestral proteins for the present day vertebrate-like NAGS and fungal NAGK, which resulted from loss of NAGK activity in the AAK domain or NAGS activity in the NAT domain, respectively.

We have determined crystal structures of both vertebrate-like and bacterial-like NAGS. Bacterial NAGS from *Neisseria gonorrhoeae* [7,8] has a hexameric molecular architecture similar to those of arginine-sensitive NAGK enzymes [9]. The six monomers form a ring with two types of intersubunit interfaces: one similar to those in arginine insensitive NAGK enzymes such as *Escherichia coli* NAGK [10] involving adjacent AAK domains and a second formed by interlacing extended N-terminal helices of adjacent subunits. The six catalytic active sites are located in six NAT domains on opposite sides of the hexameric ring. Binding of the allosteric inhibitor, L-arginine, to the AAK domain at a site similar to those

Abbreviations: AAK, amino acid kinase; CoA-NAG, CoA-*S*-acetyl-L-glutamate; GNAT, GCN5-related acetyltransferase; mmNAGS/K, *Maricaulis maris* *N*-acetyl-L-glutamate synthase/kinase; NAG, *N*-Acetyl-L-glutamate; NAGK, *N*-Acetyl-L-glutamate kinase; NAGS, *N*-acetyl-L-glutamate synthase; NAGS/K, *N*-acetyl-L-glutamate synthase/kinase; NAT, *N*-acetyltransferase; ngNAGS, *Neisseria gonorrhoeae* *N*-acetyl-L-glutamate synthase; RMSD, root mean standard deviation; xcNAGS/K, *Xanthomonas campestris* *N*-acetyl-L-glutamate synthase/kinase.

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found in arginine-sensitive NAGK enzymes, induces large conformational changes that enlarge and shorten the hexameric ring as seen in arginine-sensitive NAGK structures [9] and re-orients the NAT domain relative to the AAK domain by 109° [7]. As a result, a different surface of the NAT domain interacts with the AAK domain, the L-glutamate binding loops become disordered and enzyme activity decreases.

In contrast to the hexameric structure of bacterial-like NAGS, both bifunctional bacterial NAGS/K and NAGK in yeast exist as tetramers [5,11]. Tetramer formation involves dimeric interfaces between adjacent AAK domains and N-terminal helices as seen in bacterial-like NAGS, but the specific interactions are different. The AAK domain of yeast NAGK, without the NAT domain, is also a tetramer, implying that the AAK domain is likely to be key to tetramer formation [5]. However, in this type of NAGS, the NAT domains interact to enhance the tetramer architecture in contrast to bacterial-type NAGS in which NAT–NAT domains interactions are not seen.

The crystal structures of ngNAGS bound with various substrates that have been determined provide significant insights into the catalytic mechanism of bacterial-like NAGS enzymes. However, no other structures of NAGS/K enzymes with substrates bound have been reported, despite extensive efforts, perhaps because the affinity of these enzymes and other GCN5 related acetyltransferases (GNAT) [12] for AcCoA is significantly less that for ngNAG. However, bisubstrate analogs are often able to overcome such challenges because both substrate moieties contribute to binding.

Here we report the preparation of a bisubstrate analog, CoA-*S*-acetyl-L-glutamate (CoA-NAG), using the ngNAGS enzyme itself as the catalyst. The structure of ngNAGS complexed with the bisubstrate analog indicates that the bisubstrate analog binds to the active site similar to the way in which CoA and NAG bind separately, providing information about the intermediate reaction step. This structure, together, with biochemical analyses of several active site mutants provide further insights into the catalytic mechanism of bacterial-like NAGS enzymes. This strategy can be applied to structural and functional studies of the second type of NAGS enzymes, including human NAGS, whose deficiency causes hyperammonemia, as well as other members of the GNAT superfamily for which substrate bound structures are difficult to obtain.

2. Materials and methods

2.1. Materials and chemicals

All materials and chemicals except *N*-chloroacetyl-L-glutamate were commercially available. *N*-Chloroacetyl-L-glutamate was custom-made by Aris Pharmaceutical Inc (New Jersey).

2.2. Cloning and protein expression and purification

ngNAGS and all mutants were expressed and purified performed as described previously [8]. Briefly, the proteins were expressed in *E. coli* BL21(DE3) cells (Invitrogen) and purified with nickel affinity and DEAE columns (GE Healthcare). Protein purity was verified by SDS/PAGE gel and protein concentration was measured with a Nano-drop 1000 spectrophotometer (Thermo Scientific). An extinction coefficient of 25,900 $mol^{-1}cm^{-1}$ and molecular weight of 49.2 KDa obtained from the ExPASy web server (http://web.expasy.org/protparam/) were used to calculate protein concentrations. The protein was stored at 253 K in a buffer of 50 mM Tris–HCl, pH8.0, 50 mM NaCl, 10% glycerol, 5 mM β-mercaptoethonol, and 1 mM EDTA. Similar methods were used to over-express and purify *Maricaulis maris* NAGS (mmNAGS), *Xanthomonas campestris* NAGS (xcNAGS) and the mouse NAGS NAT domain (mNAGS), which contains mouse NAGS sequence from Met370 to Ser527.

2.3. Site-directed mutagenesis

Site-directed mutant genes of ngNAGS were created using primers containing the desired mutations (Table 1) and the QuikChange Mutagenesis Kit according to the manufacturer's protocol (Stratagene). The sequences of mutant DNA sequences were verified by DNA sequencing.

2.4. Activity assay

Enzymatic activity was assayed using the method described previously [13]. In brief, a stable isotope dilution method using liquid chromatography mass spectrometry (LC–MS) to measure NAG production was adopted. The assay was performed in a solution containing 50 mM Tris, pH 8.5, 10 mM glutamate and 2.5 mM AcCoA in a 100 μl reaction volume. The reaction was initiated by the addition of enzyme, and the mixture was incubated at 303 K for 5 min and quenched with 100 μl of 30% trichloroacetic acid containing 50 μg of *N*-acetyl-[$^{13}C_5$]glutamate as an internal standard. Precipitated protein was removed by micro-centrifugation. The supernatant (10 μl) was submitted to LC–MS (Agilent) for separation and measurement. The mobile phase consisted of 93% solvent A (1 ml trifluoroacetic acid in 1 L water) and 7% solvent B (1 ml trifluoroacetic acid in 1 L of 1:9 water/acetonitrile) and the flow rate is 0.6 ml/min. Glutamate, NAG, and ^{13}C-NAG were detected and quantified by selected ion monitoring mass spectrometry.

2.5. Detection of CoA-NAG bisubstrate analog

The LC–MS assay used to monitor activity was adapted to detect the formation of the CoA-NAG bisubstrate analog, using the similar standard reaction mixture: 100 μl of a solution containing 10 mM CoA, 10 mM *N*-chloroacetyl-L-glutamate, and 2 μg of ngNAGS in 100 mM Tris–HCl, pH 8.5. The reaction was performed at 303 K

Table 1
Diffraction data and refinement statistics.

Data collection	
Space group	P312
Wavelength (Å)	1.54178
Resolution (Å)	50–2.75
Highest resolution shell (Å)	2.8–2.75
Unit-cell parameters (Å)	a = 98.5 b = 98.5 c = 90.0
Measurements	128,602
Unique reflections	13,151 (647)
Redundancy	9.8 (9.7)
Completeness (%)	99.8 (100)[a]
R_{merg}[b]	0.087 (0.522)
$\langle I/\sigma(I)\rangle$	12.9 (1.6)
Refinement	
Reflections, working set	13,149 (1,179)
Reflections, test set	1,331 (134)
Total atom (non-H)	3.327
Protein atoms	3,227
Ligand atoms	66
Water atoms	34
R	0.189 (0.367)
R_{free}	0.271 (0.414)
Rmsd bond lengths (Å)	0.008
Rmsd bond angles (°)	1.188

[a] Figures in brackets apply to the highest-resolution shell.
[b] $R_{merg} = \Sigma_h\Sigma_i|I(h,i) - \langle I(h)\rangle|/\Sigma_h\Sigma_i I(h,i)$, where $I(h,i)$ is the intensity of the ith observation of reflection h, and $\langle I(h)\rangle$ is the average intensity of redundant measurements of reflection h.